1977

Occurence and transmission of Toxoplasma gondii in European starlings (Sturnus vulgaris) of northern California

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OCCURRENCE AND TRANSMISSION OF *TOXOPLASMA GONDII* IN EUROPEAN STARLINGS (*STURNUS VULGARIS*) OF NORTHERN CALIFORNIA

A Thesis

Presented to

the Graduate School of the

University of the Pacific

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

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May 1977

This thesis, written and submitted by

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Dated April 21, 1977

ACKNOWLEDGEMENTS

The author wishes to express sincere appreciation to: Dr. Warren J. Schneider under whose guidance, helpful criticism, and encouragement this investigation was undertaken; Drs. Fuad M. Nahhas and Francis R. Hunter for their support, advice, and helpful criticism in preparing the manuscript; Marvin Switzenberg for supplying starlings; and Darryl Behymer and David Beilstein for technical assistance which made this study possible.

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INTRODUCTION

Toxoplasma gondii, a protozoan parasite, was discovered by Nicolle and Manceaux in 1908 in Ctenodactylus gondi, a small rodent of North Africa. For nearly sixty years, the natural life cycle of T. gondii remained unknown, although some accidental laboratory infections took place and congenital transmission was known to occur. Desmonts et al. (1965) reported that transmission could occur by the ingestion of infected raw meat. Hutchison (1965) found that T. gondii could be transmitted by cat feces that had been fed to mice, and suggested transovarian transmission by the cat roundworm, Toxocara cati. Hutchison et al. (1970) however, reported that the parasite could be transmitted in cat feces which did not contain T. cati eggs. In the same article the authors described the coccidian-like nature of T. gondii. Frenkel et al. (1970) and Dubey et al. (1970) described its life cycle in cats. Frenkel (1973b) discussed the epidemiologic role of cats as the definitive or final host.

Despite these recent discoveries, the exact taxonomic status of this parasite is still disputed. Todd (1972) included Toxoplasma in the order Coccidia. This classification was based on structural similarities revealed by

the electron microscope between the merozoites and sporozoites of Toxoplasma and the coccidia and malarial parasites. Todd (1972) also stated that the intestinal stages in cats are typical of coccidian infections; schizogony and gametogony result in the production of oocysts that are structurally indistinguishable from the oocysts of Isospora bigemina, a species included in the order Coccidia. Noble and Noble (1976) stated that Toxoplasma is possibly identical with I. bigemina. Most parasitologists generally agree that T. gondii is a protozoan which multiplies by asexual division, but because of the uncertainty of its taxonomic status, it is more convenient to consider the parasite under its scientific name without assigning it to any particular class of protozoa (Beck and Barrett-Connor, 1971).

The toxoplasma parasite in the tachyzoite stage is elongate, rounded at one end, and tapered somewhat at the other end. It measures approximately five to six μm in length by two to three μm in width (Fig. 1). It is often crescentric in shape, but morphology may vary to extreme ovoid forms. T. gondii has an enteroepithelial cycle in the cat resulting in the formation of oocysts (Fig. 2). Only domestic cats and certain other members of the family Felidae have been shown to produce oocysts (Dubey and Frenkel, 1973). According to Jones (1973), the asexual and sexual stages of the enteroepithelial cycle of T. gondii

are typical of other coccidia. Atypically, however, some of these enteric stages of Toxoplasma, after ingestion of bradyzoites (Fig. 3), pass through several multiplicative generations. T. gondii also has an extraintestinal (tissue) cycle that occurs in many mammals including man and birds. The new nomenclature in existence for these extraintestinal stages consists of tachyzoites (trophozoites) and bradyzoites (cyst forms or zoites). The term "tachyzoite" is used to indicate the rapid proliferation stage of the organism found either free or intracellularly in tissue groups while the term "bradyzoite" reflects the slow replicative encysted forms (Jones, 1973). Fig 4 is a schematic representation of the life cycle; the heavy arrow points to the direction of normal development, and the light arrow in the reverse direction shows bradyzoites reverting to proliferating tacyzoites. This may occur in the same host or when encysted bradyzoites in the tissues of one animal are ingested by another mammal or bird (Frenkel, 1973b). The various stages and morphological forms of Toxoplasma have been summarized or grouped into five distinct stages by Jones (1973). Three of these stages occur in the feline enteroepithelial cycle: (1) enteroepithelial multiplicative stages (type A through E); (2) gametocyte stage; and (3) oocyst stage. The fourth and fifth stages occur in the extraintestinal (tissue) cycle in the intermediate as well as feline hosts:

(4) tissue group stage of tachyzoites formed during acute infection; and (5) tissue cyst stage of bradyzoites formed during chronic infection.

The development of T. gondii is important in understanding both the life cycle and the transmission of the organism (Fig. 5,6). Cats shed oocysts in their feces for one to two weeks during the primary infection (Frenkel, 1973b). The sporulated oocysts can remain viable in the soil for periods up to or exceeding one year depending upon temperature and soil conditions (Feldman, 1974; Frenkel et al., 1975). T. gondii is transmitted through either fecal contamination or carnivorism to a wide variety of intermediate hosts. Since T. gondii was first described by Nicolle and Manceaux in 1908, the parasite has been reported from many wild animals, birds and man. The introduction of the dye test (Sabin and Feldman, 1948) for detecting serum antibodies has been used to show the widespread and cosmopolitan distribution of the parasite. Besides fecal contamination and carnivorism, transplacental transmission is another mechanism by which intermediate hosts may become infected (Feldman, 1974). After the acute infection, the organism can survive as cysts in the body tissues for the lifetime of the host and it is the cysts in the skeletal muscles which facilitate the transfer of T. gondii by ingestion (Miller et al., 1972).

This is especially true in the case of human toxoplasmosis acquired by eating raw or undercooked meat, and possibly by handling raw meat (Jacobs et al., 1960a; Frenkel, 1973b). Jacobs et al. (1960b) also reported that mutton and pork are more commonly infected with Toxoplasma than are other meats.

After ingestion of encysted bradyzoites, the extraintestinal cycle begins with the development of tachyzoites in the lamina propria of the mammalian or avian host (Frenkel, 1973a). The extraintestinal cycle constitutes the entire cycle in nonfeline (intermediate or incomplete) hosts. In cats, it initially occurs simultaneously with the enteroepithelial cycle (Dubey and Frenkel, 1972). Tachyzoites spread from cell to cell and are disseminated to various organs in macrophages, lymphocytes, granulocytes, and in free forms in the circulation (Frenkel, 1973a). Although most of the tachyzoites are arrested in lymph nodes, liver, and lungs, a variable number enter the lymphatics and blood and are further disseminated (Fig. 7). Encysted bradyzoites begin to appear in tissues in one to two weeks as immunity develops, and they may coexist in cats with the enteroepithelial cycle (Dubey and Frenkel, 1972). Encysted bradyzoites develop intracellularly, particularly in neurons, retinal cells, and cardiac and skeletal muscle cells (Frenkel, 1973a).

A number of syndromes may result from the varied pathogenic aspects of toxoplasmosis (Jones, 1973). Most of these syndromes are inapparent infections. Frenkel (1973b) reported that one-fourth to one-half of the adults in the United States and elsewhere are asymptomatically infected. Other syndromes include lymphadenopathy or glandular toxoplasmosis; acute febrile toxoplasmosis; relapsing toxoplasmosis; encephalitis, particularly in immunosuppressed patients; toxoplasmosis during the three trimesters of pregnancy; neonatal encephalitis or jaundice; and retinochoroiditis (Frenkel, 1971). The severity of these syndromes is determined by the degree of cellular necrosis caused either directly by the number of proliferating tachyzoites or indirectly by hypersensitivity or both (Jones, 1973).

Serologic tests available for detecting toxoplasmosis include the Sabin-Feldman dye test, the indirect fluorescent-antibody (IFA) test, the complement-fixation (CF) test, the radioimmunoassay (RIA) technique, the plate hemolysin test, and the indirect hemagglutination (IHA) test. The Sabin-Feldman dye test and the IFA test detect early antibodies of the M class of immunoglobulins (IgM) and usually yield positive results in eight to ten days after Toxoplasma infection. The IFA and dye-tests also detect antibodies of the G class of immunoglobulins (IgG). The IHA

and CF test detect IgG antibodies which appear about 14 days or later after Toxoplasma infection (Krogstad et al., 1972; McKinney, 1973). CF antibody titers indicate recent or active infection and usually disappear within one to two years, whereas IFA, IHA and dye-test titers usually persist indefinitely (Krogstad et al., 1972).

Recently, much literature has appeared comparing the various serologic tests. Behymer et al. (1973) reported the IHA test to be less sensitive than the IFA test, but Araujo et al. (1971) stated that patients with antinuclear antibody have been reported as giving false-positive reactions in the IFA test. Harboe and Erichsen (1954) reported serologic nonreactivity in the dye test when experimenting with chronically infected chickens. Miller et al. (1972) also reported the failure of serologic reactivity in the dye-test in chronically infected chickens, Japanese quail, bluejays, and crows. The RIA test is a relatively recent development and the results show good correlation with the IHA test (Gehle et al., 1976). A comparison of the results of testing sera by the hemolysin, hemagglutination, and the dye-test suggested that the hemolytic antibodies were more closely related to hemagglutinating antibodies than to dye-test antibodies. Although the plate hemolysin test was developed to screen serum specimens for the presence of Toxoplasma antibodies,

it is not as sensitive nor economical as the IHA test. The IHA test is used by the United States Public Health Service Center for Disease Control (CDC) Atlanta, Georgia for the routine assay of specimens submitted for toxoplasmosis testing. It has also been used by private veterinary practitioners for testing dogs and cats because it is less expensive than the IFA procedure since it does not require the use of a fluorescent microscope (Behymer et al., 1973).

Rieman et al. (1975) have shown that enzootics of toxoplasmosis occur in nature with the role of domestic cats replaced by wild Felidae, such as bobcats, ocelot, and puma. In a recent survey of food-producing animals in California, 24 to 32% of 353 animals tested by the IHA method were seropositive for the protozoan T. gondii (Vanderwagen et al., 1974). The investigators noted a dearth of domestic cats, considered to be the primary amplifying host of toxoplasmosis, in several areas from which seropositive animals originated. They suggested wildlife as a potential reservoir of T. gondii. Wildlife species could be an important link in the transmission of T. gondii to livestock on the open range. There is a possibility that one such intermediate host or reservoir is the European Starling (Sturnus vulgaris), a common species of wild aves in California. Little research has been done to determine the role of birds in T. gondii transmission,

and none has been done to date on starlings. Jacobs and Melton (1966) found a total of 62 domestic chicken hens positive for T. gondii cysts. Miller et al. (1972) isolated Toxoplasma from chickens, Japanese quail, bluejays, and crows. They also found that organs positive for Toxoplasma were brain, heart, spleen, kidney, skeletal muscle, and adrenal, with the brain and heart having the greatest concentrations of organisms. Wallace (1971) infected wild rats, mongooses, doves, pigeons, chickens, sparrows, and Brazilian Cardinals with T. gondii; infection in chickens and crows was demonstrated by organ feeding to mice.

A comprehensive study was done by Schneider (1969) concerning the role of European Starlings in transmission of livestock and human diseases. It was reported that a total of 34 parasites (16 ectoparasites and 18 endoparasites) had been found in starlings in North America, but no mention was made of T. gondii.

This research was performed to determine; (1) the prevalence of Toxoplasma antibodies among starlings in Northern California, (2) if such starlings can transmit the disease, and (3) if tissues from seropositive starlings contain encysted forms of T. gondii.

MATERIALS and METHODS

Experimental subjects. A total of 563 native fledgling starlings, Sturnus vulgaris, were trapped and supplied by Mr. Marvin Switzenberg, Agricultural Commissioner in charge of the San Joaquin County, California Starling Control Project during the period September 1975 through October 1976. The starlings were collected from stationary traps placed in vineyards, orchards and a dairy operation on nine ranches located within a ten mile radius near Lodi, California.

The 141 birds trapped in September and October 1975 were tested only for serum antibodies against T. gondii utilizing the indirect hemagglutination (IHA) technique. Beginning in November 1975, all birds trapped were tested for serum antibodies, and tissues (brain and heart) from ten seropositive starlings were used in transmission experiments.

Indirect hemagglutination test. Blood samples were collected from starlings via cardiac puncture. After clotting for ten minutes, the blood was centrifuged at 1000 x G for ten minutes using a six place, safety head centrifuge (Scientific Products, McGraw Park, Illinois). The supernatant serum was decanted into a sterile polyethylene storage

container and within 48 hours was tested for specific antibodies against T. gondii by the microtiter system of the IHA test. The test kit was purchased from Industrial Biological Laboratories, Inc. (Rockville, Maryland). The IHA test employs stabilized sheep red cells sensitized with a soluble extract of T. gondii grown intraperitoneally (IP) in mice or in tissue cultures. Test serum containing T. gondii antibodies agglutinates the antigen-coated sheep redcells which then settle into a distinctive mat covering the bottom of the microtitration test well. Starlings with end titers of 1:64 or greater were considered "positive". The 1:64 dilution has been established by the CDC as the lowest dilution considered significant in sera tested by the IHA technique. The significance of titers lower than 1:64 has not been established. Details of the test as followed in this investigation are described in Appendix 1.

Transmission experiments. Beginning in November 1975, bird carcasses were retained at 4C after collection of blood samples. Within 24 hours after bleeding, one gram of cardiac and brain tissue from each seropositive starling (a total of ten birds) was used to make a 20% suspension in 0.9% sterile saline. The remaining cardiac and brain tissue was preserved in 10% formalin. One ml of the suspension was inoculated IP into each of two Sprague-Dawley rats and a third rat was inoculated with sterile saline as

a control. Before the rats were inoculated, blood samples were found to be free of antibodies against T. gondii. Blood samples were obtained from the rats by removing the tip of the tail with a razor blade. The blood was collected in a capillary tube, allowed to clot for ten minutes and centrifuged at 1000 x G for ten minutes using a 24-place, micro-hematocrit centrifuge (Scientific Products, McGraw Park, Illinois). Twenty-two and 82 days after inoculation, blood samples were again taken from the rats via the tail and tested for specific antibodies to T. gondii. A total of twenty rats including controls were used. Eighty-two days after inoculation, the rats were necropsied, the brain and heart tissue was preserved in 10% formalin for histologic studies and smears of peritoneal exudate were stained with Giemsa and studied under a high dry objective.

Absorption of nonspecific agglutinins. A 10% suspension of stabilized sheep red cells prepared from the same lot as the antigen-sensitized red cells was used to remove the nonspecific agglutinins, such as heterophile antibodies, from the "positive" test serum. According to the Industrial Biological Laboratories, Inc., the absorbent removes cell-specific inhibitors, permitting the serum to be retested without interference from them. All "positive" sera were retested using the absorbent reagent in the IHA test and starlings with end titers of 1:64 or greater were considered true positives. Detailed procedure of this

absorption technique is given in Appendix 2.

Tissue sectioning and staining. The preserved cardiac and brain tissue from ten seropositive starlings (S-208 through S-541 in Table 1) and twenty Sprague-Dawley rats used in the transmission experiments were processed and stained. A 0.3 to 0.5 cm representative cross section, i.e. gross section was made from each tissue. The tissue sections were then processed with an automatic processor which utilized a vacuum system to insure maximum infiltration of all solutions. Tissues were fixed in buffered formalin, dehydrated in alcohol, cleared in xylene, and infiltrated with paraffin. Details of tissue processing are described in Appendix 3.

After infiltration, the tissues were cooled at 0 to -5C and sectioned at six μm thickness. The section placed on the slide for staining was randomly selected from the first ribbon yielding a complete representation of the tissue. The slide was dried at 60C for one hour, cooled at 25C ± 5C, and stained at 25C ± 5C. A modification of Mayer's hematoxylin-eosin method was used which involved clearing, hydrating, staining with Mayer's modified hematoxylin, counterstaining with eosin, dehydrating, and reclearing (Molnar, 1976). Detailed staining procedure with the modified Mayer's hematoxylin-eosin method is given in Appendix 4.

Upon completion of the final clearing, one drop of permount was added to the slide and a cover slip placed over the tissue. The slide was allowed to dry for at least 24 hours and studied using the oil immersion objective for the presence of bradyzoites.

Calculation of prevalence rate. The prevalence rate is defined as the number of existing cases of a disease at a point in time divided by the total population at that particular point in time (Mausner and Bahn, 1974). The prevalence rate in this investigation was calculated by dividing the number of seropositive starlings by the number of starlings and expressed as a percentage.

RESULTS

Of 563 starlings collected, 34 birds (6.0%) were initially seropositive for antibodies against T. gondii using the IHA test. After retesting the sera in order to absorb nonspecific agglutinins, 27 birds (4.8%) remained seropositive and were considered true positives. Of the seropositive starlings, 19 had end titers of 1:64, two had end titers of 1:128, four had end titers of 1:256, and two had end titers of 1:512 (Table 1).

The number of seropositive starlings and the number of starlings collected were recorded with respect to location and time of collection so that prevalence rates could be calculated for each variable. Table 2 gives the prevalence rate of starlings seropositive for T. gondii based on source of starlings. Ranch 1 had the highest prevalence rate (17%) while Ranch 5 and Ranch 8 had the lowest prevalence rates (0%). Table 3 gives the prevalence rate of starlings seropositive for T. gondii based on time trapped and tested. The months from May through October had the highest prevalence rates. September (1975) had the highest prevalence rate (15%) followed by May (1976) with a prevalence rate of 13%. The period from November through April

had the lowest prevalence rates. All the months within this period had 0% prevalence rates with the exception of January (1976) which had a prevalence rate of 3%.

Tissues from ten seropositive starlings (S-208 through S-541) were used in the transmission experiments. Twenty rats inoculated with tissues from the seropositive starlings were seronegative 22 and 82 days after inoculation. Peritoneal smears made from these rats at post-inoculation day 82 were also negative for T. gondii.

Slides made from the brain and cardiac tissue of the ten seropositive birds and the twenty rats inoculated in the transmission experiments were carefully scanned for Toxoplasma bradyzoites. The rat tissues were negative for T. gondii bradyzoites while one starling contained structures resembling T. gondii cysts. Three of these structures were located in the brain tissue of starling S-208.

DISCUSSION

The overall prevalence rate of antibody-positive serum from starlings was 4.8% of 563 tested. The prevalence rate was also calculated with respect to location and time. The various ranches from which starlings were obtained are located within a ten mile radius near Lodi, California. No significant conclusions were reached regarding the location of seropositive starlings because of two factors: (1) birds were not collected at a uniform rate from each ranch during the test period; and (2) birds probably ranged throughout the collection area during the test period.

Time was the most significant factor correlated with the prevalence of T. gondii antibody. Starlings were trapped and tested for T. gondii antibodies during fourteen consecutive months (Table 3). Differences in prevalence rates coincided with two semiannual periods based on climatic and seasonal characteristics. The May-October period was characterized by higher temperatures and little or no rainfall as compared to the November-April period which was characterized by lower temperatures and greater rainfall. The two semiannual periods were

compared statistically using the Student's t test and the prevalence rates were significantly higher during the May-October period than during the November-April period. The difference was significant at the 0.005 level ($t = 4.31$).

The difference in prevalence rates may be due to climatic and seasonal fluctuation between the semiannual periods and/or farming practices in the Lodi area of San Joaquin County. During the warm, dry months of the year, starlings feed on grains and grasses and come into contact with the soil more often than they do during the cold, rainy months. When soil contact is enhanced, the probability of ingesting viable oocysts and becoming infected with *T. gondii* is increased. The two highest prevalence rates occurred in May and September and correspond to the farming practices in the Lodi area. Farmers disc the soil in the Spring in preparation for planting and they usually disc the soil again in the Fall. Large numbers of starlings have been observed feeding in the soil at these times, thereby increasing the opportunity for oocyst ingestion.

Besides time and location, host characteristics are also important factors in epidemiologic studies. Age is usually the most important host characteristic. Fledglings (less than one year old) were trapped more frequently than adult starlings because they tended to flock together

more often and were trapped quite readily. Virtually all the birds trapped and tested for antibodies against T. gondii were fledglings; therefore, it was not possible to correlate age with the prevalence of T. gondii antibody. Titers of 1:64 and greater in the fledglings tested probably indicated a recent or an ongoing parasitic infection of T. gondii.

The fact that rats in the transmission experiments remained seronegative and had no signs of T. gondii infection may have been due to one of three reasons: (1) the positive reactions of the starlings to the IHA test were "false-positives", i.e. caused by a cross-reacting organism or non-specific substance not eliminated during the absorption phase of the IHA test; (2) the starlings were infective, but the heart and brain tissues used did not contain infective stages; or (3) the technique of organ passage from starlings to rats was not suitable due to the differences in metabolic rate and body temperature, i.e. strains of T. gondii may exist which are specific for starlings and unable to survive in rats.

Structures resembling T. gondii bradyzoites were found in one starling suggesting that the European Starling may serve as an intermediate host for T. gondii. Since starlings may harbor Toxoplasma, they could serve as an important wildlife reservoir. The rural Lodi area has a high population of domestic and feral cats and starlings

could serve as another source for feline infection. This source of infection, coupled with the fact that starlings are omnivorous and exhibit cannibalistic behavior, may contribute to the perpetuation of T. gondii in the wild. Therefore, starlings may be a link in the transmission of toxoplasmosis to livestock on the open range.

SUMMARY

The sera of 563 fledgling starlings trapped during a 14 month period near Lodi, California were examined for antibodies to Toxoplasma gondii, using the microtiter indirect hemagglutination method. Titers of 1:64 to 1:512 were found in 4.8% of the birds. Starlings collected during May through October had a higher antibody prevalence rate than those collected during November through April. Heart and brain suspensions from ten seropositive starlings were inoculated into twenty rats, which remained seronegative for T. gondii antibodies when tested at 22 and 82 days post-inoculation. Peritoneal smears and tissue sections made from these rats at post-inoculation day 82 were negative for T. gondii. The tissue section from one seropositive starling contained structures resembling T. gondii bradyzoites.

Table 1. Starlings seropositive for *Toxoplasma gondii* with corresponding end titers.

Seropositive Starling	End Titer
S-011	1:256
028	1:64
033	1:64
039	1:64
045	1:64
046	1:64
053	1:64
054	1:64
055	1:64
065	1:64
068	1:64
070	1:64
098	1:256
099	1:512
100	1:128
126	1:256
129	1:64
208	1:64
212	1:64
374	1:512
383	1:64
384	1:64
385	1:64
411	1:64
504	1:128
511	1:256
541	1:64

Table II. Prevalence rate of starlings seropositive for *Toxoplasma gondii* based on source of starlings.

Location	Number of Starlings Tested	Number of Seropositive Starlings	% Prevalence of Antibodies
Ranch 1	46	8	17
Ranch 2	35	4	11
Ranch 3	45	3	7
Ranch 4	39	2	5
Ranch 5	56	0	0
Ranch 6	102	6	6
Ranch 7	31	1	3
Ranch 8	60	0	0
Dairy 1	149	3	2

Table III. Prevalence rate of starlings seropositive for *Toxoplasma gondii*, based on time trapped and tested.

Month	Number of Starlings Tested	Seropositive Starlings	% Prevalence of Antibodies
September 1975	81	12	15
October 1975	60	5	8
November 1975	39	0	0
December 1975	25	0	0
January 1976	67	2	3
February 1976	29	0	0
March 1976	36	0	0
April 1976	24	0	0
May 1976	30	4	13
June 1976	41	1	2
July 1976	39	0	0
August 1976	21	0	0
September 1976	38	2	5
October 1976	33	1	3

Fig. 1. Toxoplasma gondii tachyzoite X 10,000.

Fig. 2. Toxoplasma gondii oocyst X 2,000.

Fig. 3. Structure resembling Toxoplasma gondii bradyzoite X 2,000 drawn from brain tissue of starling S-208.

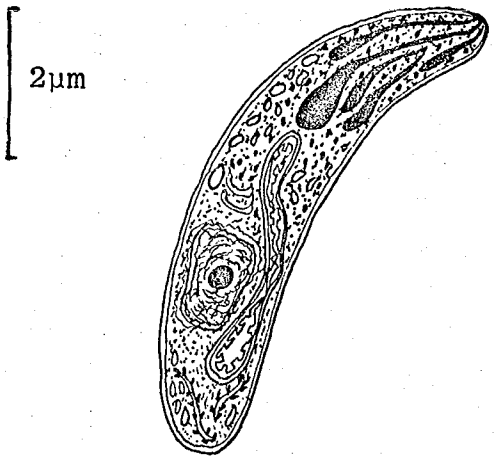

Figure 1

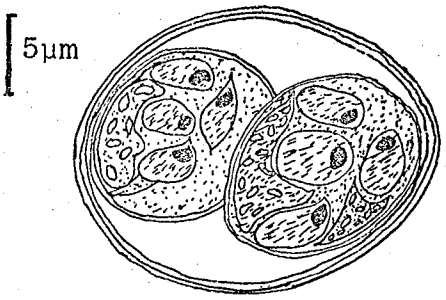

Figure 2

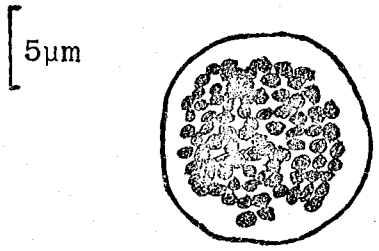

Figure 3

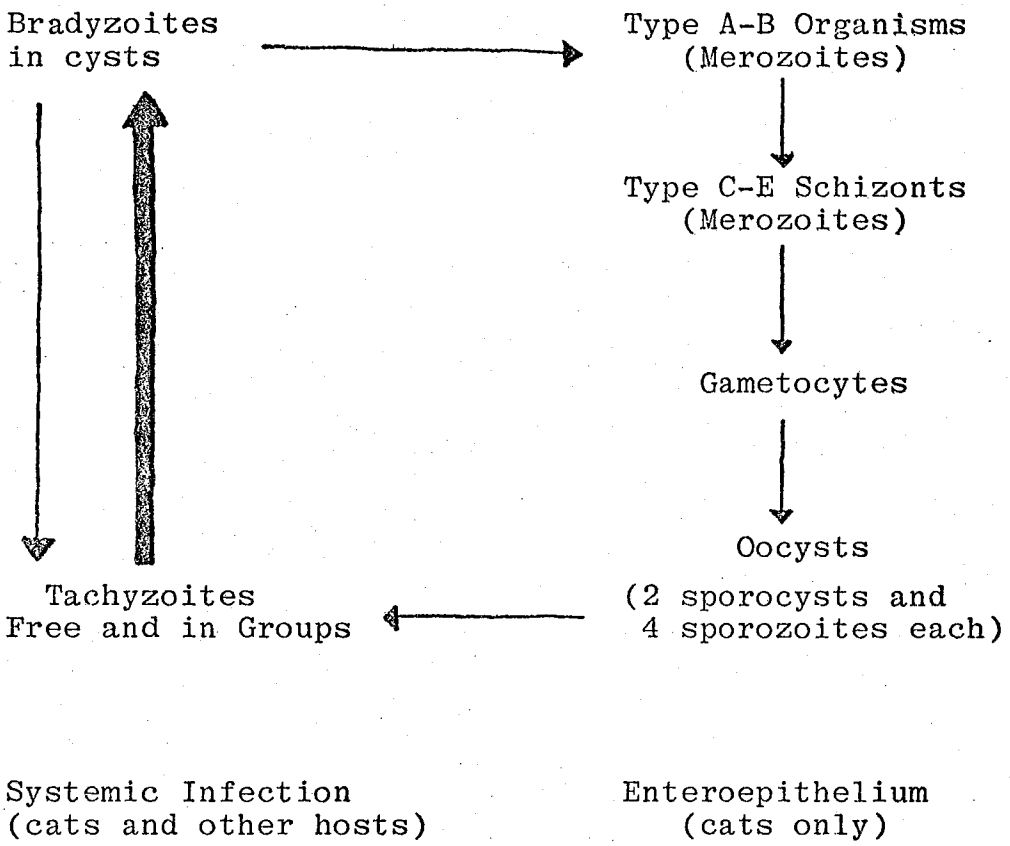

Fig. 4. The sequence of development of Toxoplasma gondii. (From Jones, S. R.: J. Amer. Vet. Med. Assoc. 163:1038, 1973.)

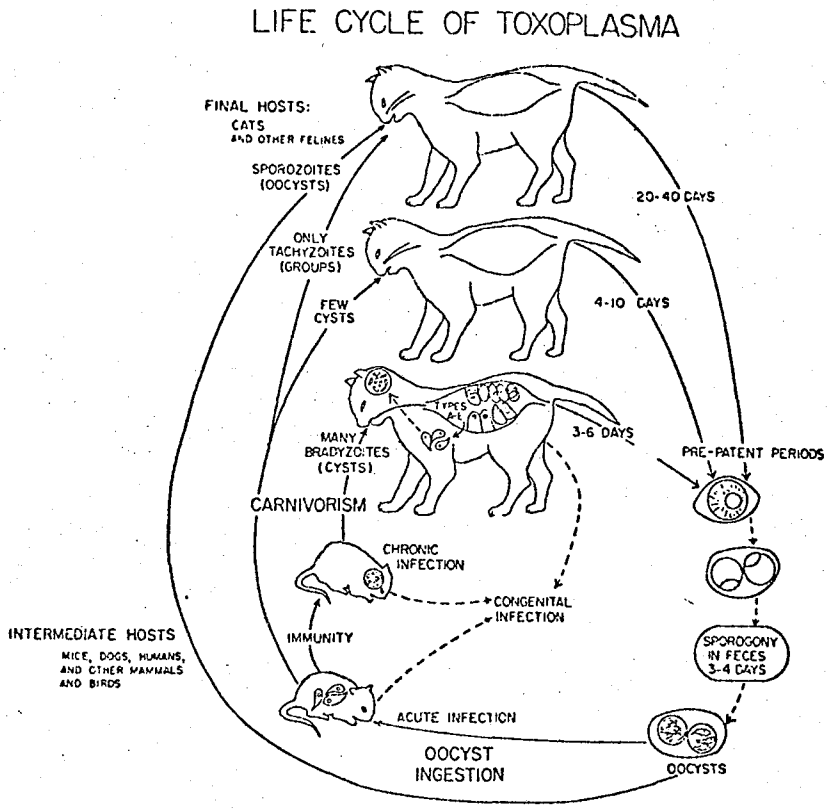

Fig. 5. Life cycle of Toxoplasma showing means of transmission by oocysts from cat feces and by ingestion of trophozoites or cysts from intermediate hosts. Days indicated represent time from ingestion of Toxoplasma by cats to the shedding of oocysts. Mice are shown to represent the many mammals and birds that can serve as incomplete or intermediate hosts and among which infection may spread, usually by carnivorism.
(From Noble, E. R. and Noble, G. A.: Parasitology. Philadelphia, Lea and Febiger, 1976, p. 85.)

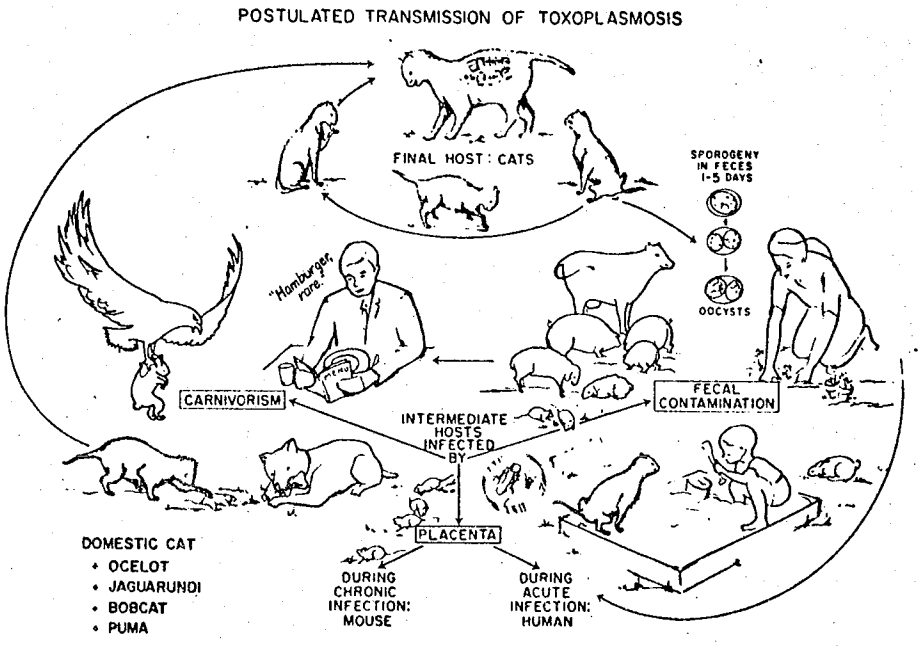

Fig. 6. Transmission of toxoplasmosis. Oocysts are shed in the feces of cats and other Felidae, and after sporulation are infectious to a great variety of hosts. Direct fecal contamination and transport hosts, such as flies and cockroaches, are important. Carnivorism and transplacental transmission are also important.
(From Frenkel, J. K.: Bioscience 23: 345, 1973.)

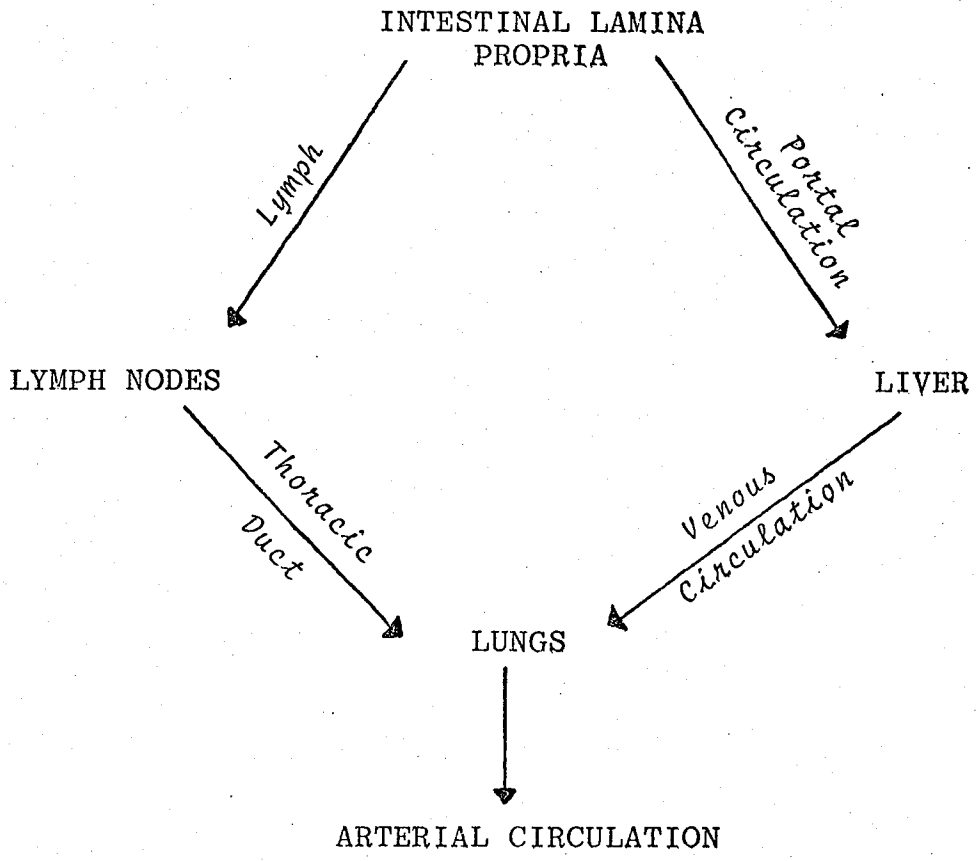

Fig. 7. Routes of dissemination. After ingestion of encysted bradyzoites or sporulated oocysts by any mammalian or avian host, extraintestinal infection originates in the intestinal lamina propria by the proliferation of tachyzoites. Lymphatic and venous circulation carry tachyzoites through the heart to the lungs, where they may be further disseminated via arterial circulation. (From Jones, S. R.: J. Amer. Vet. Med. Assoc. 173: 1039, 1973.)

APPENDIX 1

INDIRECT HEMAGGLUTINATION TEST

(1) Prepare and test controls. Positive and negative control sera are provided with the Toxo-IHA kit to monitor test accuracy and reproducibility. These are whole sera and must be prepared, diluted, and handled in the same manner as sample sera. The positive and negative controls must be tested each time the Toxo-IHA kit is used. Failure to test controls makes it difficult to recognize false-positive or false-negative results.

(2) Using a glassware marking pen, mark the clear, disposable U-bottom microdilution trays with sample identification numbers.

(3) Prepare a 1:64 dilution of the test serum by adding 0.025ml of diluent to each well of the test tray. Add 0.05ml of the test serum to the first well of the test tray. With the microtitration diluters, 0.025ml capacity, mix the serum and diluent in well No. 1 and transfer 0.025ml to well No. 2. Repeat this step through well No. 6 for each serum sample.

(4) Gently swirl the bottle of sensitized cells until the cells are completely resuspended and no sediment is visible on the bottom of the bottle.

(5) Using the bottle's calibrated dropper tip, carefully squeeze a drop (0.05ml) of sensitized cells into well No. 6 (1:64 dilution).

(6) Shake the tray by gently tapping it several times against the side of one hand to insure complete mixing of test reagents.

(7) Incubate the tray at room temperature (25C $\pm$ 5C) for two to three hours or until the cells have settled into distinct patterns. Do not pick up or otherwise disturb the tray during the incubation period.

(8) After incubation, read the test results.

(a) positive reactions are those which produce a +2, +3 or +4 hemagglutination pattern. A smooth mat of cells covering the entire bottom of the well with the edges sometimes folded is characteristic of a +4 hemagglutination pattern. The pattern of a +3 reaction is a smooth mat of cells covering less area of the well and a +2 reaction pattern is a smooth mat of cells surrounded by a thin red circle.

(b) negative reactions are those which produce a +1, +/-, or - hemagglutination pattern. The hemagglutination pattern of a +1 reaction is a small mat of cells surrounded by a heavy red circle, the pattern of a +/- reaction is a button of cells having a small "hole" in the center, and a compact button of cells sometimes having a very small hole in the center is characteristic of a minus (-) reaction.

APPENDIX 2

ABSORPTION OF NONSPECIFIC AGGLUTININS.

(1) Gently swirl the bottle of absorbent until the cells are completely resuspended and no sediment is visible on the bottom of the bottle.

(2) Using a one ml serological pipet, dispense 0.69ml of absorbent into a test tube.

(3) Using a 0.1ml serological pipet, add 0.02ml of undiluted test serum to the test tube.

(4) Mix by swirling the test tube.

(5) Incubate the mixture at room temperature (25C $\pm$ 5C) for one hour.

(6) Centrifuge the tube at 900 X G for ten minutes.

(7) The supernatant fluid provides a 1:32 dilution of the test serum. Mix this dilution with an equal part of diluent to give an initial test dilution of 1:64 and retest according to the instructions given for the IHA test in Appendix 1.

APPENDIX 3

TISSUE PROCESSING

Gross sections were put into metal casettes and fixed, dehydrated, and cleared in the automatic processor at 35C while the infiltration of paraffin was done at 61.5C. The gross sections were passed through a series of solutions:

(1)	10% buffered formalin	1 hour
(2)	10% buffered formalin	1 hour
(3)	70% isopropyl alcohol	1 hour
(4)	95% isopropyl alcohol	1 hour
(5)	95% isopropyl alcohol	1 hour
(6)	100% isopropyl alcohol	1 hour
(7)	100% isopropyl alcohol	1 hour
(8)	100% isopropyl alcohol	1 hour
(9)	xylene	1 hour
(10)	xylene	1 hour
(11)	paraffin	1 hour
(12)	paraffin	1 hour

APPENDIX 4

TISSUE STAINING

Mayer's hematoxylin-eosin method of staining was modified to provide several distinct features: solutions are more concentrated; chloral hydrate has been added to the hematoxylin solution to act as a preservative; the color acid of eosin is used as the counterstain; the staining is faster, and there is no need to change the solution. When the stain starts evaporating, it is diluted to the original volume with fresh solution. The staining solution remains stable and can be used constantly without changing for over a year. In contrast to Mayer's solution, which allows slide colors to fade in a short time, this modified solution produces slide colors that remain stable indefinitely. In addition, unlike Lillie's modified solution, it can be used on all fixatives and tissues including cadavers and bone sections, and for special photography studies. A well-differentiated stain with blue nuclei and bright rose background is obtained.

Slides with the sectioned tissue were passed through a series of staining dishes in order to clear, hydrate, stain, counterstain, dehydrate, and reclear as follows:

(1)	xylene	2 min.
(2)	xylene	2 min.
(3)	100% isopropyl alcohol	1 min.
(4)	100% isopropyl alcohol	1 min.
(5)	100% isopropyl alcohol	1 min.
(6)	deionized water	4 min.
(7)	modified Mayer's hematoxylin	2-3 min.
(8)	running tap water	5 min.
(9)	70% isopropyl alcohol	1 min.
(10)	alcoholic eosin	1-1.5 min.
(11)	95% isopropyl alcohol	1 min.
(12)	95% isopropyl alcohol	1 min.
(13)	100% isopropyl alcohol	1 min.
(14)	100% isopropyl alcohol	2 min.
(15)	xylene	2 min.
(16)	xylene	2 min.
(17)	xylene	2 min.

Modified Mayer's Hematoxylin

Hematoxylin.....	4.0gm
Distilled water.....	1000.0ml
Sodium iodate.....	0.3gm
Ammonium or potassium alum.....	50.0gm
Citric acid.....	1.5gm
Chloral hydrate.....	75.0gm

Dissolve alum in water (do not use heat); add hematoxylin, iodate, citric acid, and chloral hydrate, in order. Filter hematoxylin solution through coarse filter paper.

Eosin (Color Acid) - stock

Eosin Y (NE 39)..... 22.0gm
Distilled water..... 500.0ml
Hydrochloric acid, concentrated.... 12.0ml

Dissolve eosin completely in distilled water, then add the concentrated hydrochloric acid. Allow to settle overnight and filter. Wash the precipitate on the paper with distilled water until only a pink tint appears in the wash water. Dry the precipitate on the filter paper at 37-40C for 12-24 hours.

Eosin (Color Acid) - working

Dissolve 15 grams of the dry stain in 1000ml of 95% ethyl alcohol. Let solution settle two to three hours, and filter into staining container.

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